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Patents ADP number *(if you know it)* 07531635001.

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4. Title of the invention

FIBRIN FRAGMENTS AND THERAPEUTIC TARGETS

5. Name of your agent *(if you have one)*

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Fibrin fragments and therapeutic targets

This invention describes the creation and application of therapeutic agents derived from existing knowledge of the active site on fibrin fragment E and its cell surface receptor. These agents will include, but not be restricted to, antibodies, antibody-like molecules, synthetic protein analogues and other recognition molecules which mimic or block the interaction of fibrin fragment E with its receptor. The therapeutic agents proposed are of wide application to many types of disease problems in man and animals that share the same underlying common features due to basic pathological events consequent to fibrin deposition and lysis. Such molecules may also be linked to other agents such as cytotoxic drugs or agents used for anatomical or biochemical imaging.

Background

(a) Basic Pathology of Chronic Inflammation, Healing and Repair:

Fibrinogen is the major circulating plasma protein involved in blood clot formation. Activation of the clotting enzyme cascade, by for example injury or inflammation, results in conversion of prothrombin to thrombin which cleaves two small fibrinopeptides (A and B) from each soluble fibrinogen molecule to give fibrin monomer. Cross linkage of monomers is the final step of the coagulation system that gives solid fibrin. Whole blood includes platelets and forms blood clot in wounds, and termed thrombus when in abnormal arteries and veins: inflammatory exudate is platelet free and forms fibrin alone.

Fibrin deposition and degradation is a major feature of the pathology of acute and chronic inflammation at any site in the body and regardless of the underlying disease aetiology. This process is apparent at the histological level in the healing wound, the organising thrombus, the advanced atherosclerotic plaque, and many other types of pathological lesions including the growing edge of some types of cancer. The fibrin mesh provides a provisional matrix for cell ingrowth, being progressively invaded in wound healing by inflammatory cells (macrophages), new small blood vessels (capillary buds), connective tissue cells (fibroblasts) and the epidermis (squamous epithelium). In the context of the large arteries subject to atherosclerosis, the endothelium of the luminal surface and the smooth muscle cells of the vascular wall invade the fibrin mesh. Secretion of plasminogen activator is the common factor that provides controlled lysis of the fibrin substratum via plasmin degradation, releasing fibrin degradation products. Fibrin degradation products are composed of combinations of two moieties termed fragments D and E. Eventually the fibrin present is replaced by new cells and matrix forming new tissue. These basic features apply to many types of human and animal disease.

(b) Fibrin Degradation Products:

Although fibrin may be a factor common to many pathologies involving cell proliferation, it has generally been assumed that its main function was to provide an inert physical matrix to support cell movement. However there has been evidence for some time that fibrinopeptides and fibrin degradation products have biological activity particularly as soluble mediators of chemotaxis, the phenomenon of directional cell movement (1). We proposed that fibrin degradation products were a major pathological growth factor common to all sites of chronic inflammation. The chick chorioallantoic membrane is a standard in vivo test model for detection of angiogenic growth factors and this was used to demonstrate that fibrin degradation products were indeed angiogenic (2), had stimulatory effects on collagen-synthesis (3), and that specifically fibrin fragment E was the active component (4).

(c) The Relevance of Fibrin Deposition and Lysis to a Wide Spectrum of Human Diseases:

Angiogenesis and Wound Healing

As explained above, fibrin deposition and lysis are essential features, as demonstrated by abnormalities of healing in the plasminogen and fibrinogen knockout mouse models (5, 6). We have shown in a mouse incised wound model that the peak of angiogenic activity in simple wound extracts occurs at day 3, preceding the peak of wound vascular density at day 5 (7). Technical difficulties have been explained and overcome recently showing that the bulk of the angiogenic activity is attributable to fibrin fragment E (8). Specific anti fibrin E antibodies were used to block extract activity by admixture before testing on the chick chorioallantoic membrane.

Vascular Restenosis:

Angioplasty is a commonly used technique to dilate the narrowed segments of atherosclerotic artery, especially the coronary arteries, in patients with vascular insufficiency. Many patients unfortunately require re-operation within months due to arterial narrowing (restenosis) from smooth muscle cell proliferation. The likelihood of clinically significant post-angioplasty restenosis has been generally understood to be predicted to a fair extent by the amount of blood clot at the angioplasty site. The antithrombin drug hirudin has been partially successful in reducing restenosis experimentally and in man (9), but it has been unclear whether this is attributable to clot reduction or prevention of direct thrombin stimulation of smooth muscle cell proliferation via the PAR-1 thrombin receptor (10).

However it is now apparent that the PAR-1 knockout mouse is normal and has near normal wound healing (11), and that intimal hyperplasia is not inhibited by an antisense thrombin receptor oligodeoxynucleotide following carotid injury in the rabbit (12). An alternative candidate that is both thrombin and plasmin dependant, but not thrombin receptor dependant is fibrin fragment E. Fibrin degradation products are abundant at sites of healing and repair, including sites of vascular injury and in extracts of proliferative types of human atherosclerotic plaque. We have shown that fibrin fragment E stimulates smooth muscle cell proliferation and outgrowth from aortic media explants in culture (13). This occurs in serum rich culture in which thrombin is inactive.

Atherogenesis:

The link between abnormalities of the coagulation system and thrombosis of a coronary artery is well established not only in terms of the actual clot but in terms of risk of myocardial infarction in human populations. In many prospective studies, the plasma levels or activities of coagulation factors such as fibrinogen, factor VIIa and fibrin degradation products (D dimer assays) have been shown to be predictive risk factors for myocardial infarction, and other vascular events such as stroke and progressive peripheral vascular disease (14, 15, 16). A substantial proportion of the risk due to cigarette smoking is attributable to raised fibrinogen. The mode of interaction of this risk factor with the actual lesions of atherosclerosis, the atherosclerotic plaques within the artery wall, remains unexplained, but the end result, accumulation of fibrin admixed with the lipid core of the plaque and forming overlying thrombus in minor and major terminal events is well established.

The major pathogenic feature of atherogenesis is the response to fibrin and lipid accumulation by the smooth muscle cells of the arterial wall. Smooth muscle cell proliferation has long been recognised as the key event in plaque development, as it is in the more acute lesion of post angioplasty restenosis (17). Atherosclerotic lesions can be divided into several types, the earliest thought to be the gelatinous lesions, the precursors of the fibrous plaques. The early gelatinous lesions contain little lipid but significant amounts of fibrin related antigens (FRA) (18). In the more advanced lesions, fibrin is deposited in layers suggesting repeated thrombotic

episodes (19). In all lesions the FRA are largely derived from cross-linked fibrin not fibrinogen suggesting continuous deposition and lysis of fibrin (20).

Soluble extracts of intima from active types of lesions from human autopsy and surgical material have been shown by us to stimulate cell proliferation in the in vivo test model already mentioned, the chick chorioallantoic membrane (21). This work was extended to show that for a short series of stimulatory extracts, the bulk of the activity was removed by passing each through an affinity column containing antifibrin(ogen) antibody (22). Selective removal was again achieved with a bound specific anti fragment E antibody, but not with a bound anti fragment D antibody.

Other Relevant Diseases:-

Rheumatoid Arthritis: This chronic disease of joints is of unknown cause but is known to be driven by the immune system. This causes episodic inflammation of the synovial lining of the joint, with deposition of fibrin which becomes organised by fibrovascular ingrowth, termed pannus, forming a membrane rich in inflammatory cells. This extends over the joint cartilage, releasing proteolytic enzymes that digest and gradually destroy the joint surface causing pain and immobility. Anti inflammatory drugs help symptoms but do not arrest disease progress significantly. Prevention of pannus extension during acute episodes may well be advantageous.

Diabetes: Diabetic retinopathy is the major cause of blindness in the Western world. It is due to narrowing of the microvascular blood supply vessels within the eye as elsewhere in the body in poorly controlled diabetes mellitus. It is characterised by proliferation of leaky new blood vessels in response to ischaemic areas of retina. The combination of fibrovascular proliferation and contraction involves the vitreous and distorts and destroys the retina. Fibrin deposition and degradation are likely to contribute to this disadvantageous instance of normal healing and repair.

Renal Disease: Acute and focal glomerulonephritis are characterised by deposition of both immune complexes and fibrin in the glomerulus with associated inflammatory cells. There is resultant cellular proliferation leading to glomerulosclerosis and permanent loss of function with renal failure in many cases. Anti-inflammatory drugs could be supplemented with the agents proposed to inhibit cell proliferation.

Tumour Growth and Metastasis: For a malignant, invasive epithelial tumour of any type to grow more than 1mm and invade surrounding normal tissues, there is an absolute requirement for recruitment of a new blood supply. This phenomenon of tumour angiogenesis provides a target for therapeutic anti cancer intervention. These new small capillary vessels are leaky, and plasma proteins including fibrinogen are abundant in adjacent connective tissue at the tumour edge. Although Dvorak coined the appealing phrase " the wound that does not heal" to describe the moving edge of stroma surrounding the invasive tumour, his early assertions that fibrin was deposited, as in a wound (23), have not held up to scrutiny with newer fibrin specific antibodies for immunostaining. Most tumour types do not display a complete set of procoagulant factors for this to happen. However there are two major exceptions, oat cell carcinoma of lung and clear cell carcinoma of kidney (24, 25). These two tumour types are common, are extremely vascular, show fibrin deposition, and are hard to treat once spread by metastases from the site of origin has occurred. Leo Zacharski has shown some modest improvement in survival in a trial of terminally ill patients with oat cell carcinoma with the drug warfarin which inhibits clotting (26). One problem with such drugs is that complete inhibition is never achieved because of very real risks of major haemorrhage. Inhibition of the fibrin E stimulatory contribution not to tumour growth but to tumour angiogenesis may be a useful adjunct to the current partially effective treatments by chemotherapy and radiotherapy.

Fibrin Glues for Surgery: Modification of fibrin glues to promote or inhibit the cell proliferation induced by the fibrin degradation products can be achieved by preparation and admixture with promoters or inhibitors of the site of interaction of fibrin E. These glues are increasingly used for a wide variety of operations where sutures are impractical.

Clinical Problems:

In all these examples of disease, the clinical aim would be to prevent the initiating cause but this is often not possible. Selective inhibition of angiogenesis and cell proliferation would be highly desirable. At the same time, it would be preferable to avoid interference with other aspects of the normal inflammatory response, and with blood clotting and fibrinolysis. However clinical manipulation at one site may conflict with clinical problems at other sites. For example, systemic administration of an antiangiogenic drug to inhibit tumour growth may adversely affect normal healing and repair of a healing skin wound, or a peptic ulcer of stomach.

The severity of potential limitations depends on the therapeutic margin of treatment success over unwanted side effects. Short term treatment and localised treatment offer solutions to some problems but not all. We propose that a further way round such problems is to utilise protective or therapeutic treatments at the other sites and conditions at risk of collateral damage. This is our rationale for development of not only drugs that block angiogenesis, smooth muscle cell proliferation, and other cell type proliferation, but also drug antagonists for local protective application, and also drug agonists that stimulate angiogenesis and the normal cell proliferative response. Combinations of systemic and local administration may become feasible and effective.

The Essential Features of the Invention:

Fibrin E receptor:

According therefore to the first aspect of the present invention, the cell surface receptor for fibrin fragment E which we have identified can be used to select drugs or other agents which will prevent, or in some cases stimulate, the fibrin fragment E response. Cell binding of the fragment E was demonstrated using ligand blotting of SDS-PAGE gels of cell membranes from chick fibroblasts. A single band (approximately 66kD) can be seen on the blot (fig1). This has also been supported by gels of membranes previously challenged with fibrin fragment E, resulting in a band of approximately 122kD (fig2), which is consistent with fragment E (55kD) bound to a membrane fragment of 66kD. ELISA binding studies, and by immunohistochemistry of a range of fibroblast cell types (chick, human and mouse) (Fig. 3A and 3B). This receptor may be of considerable importance in the control of cell proliferation seen in atherosclerosis, and particularly in post angioplasty restenosis.

Anti-fibrin E blocking antibodies:

Another feature of the present invention is the generation of antifragment E antibodies which prevent the binding of the fibrin fragment E to its receptor. Polyclonal antibodies which block the cell proliferative activity of fibrin fragment E can be raised by injection of the whole fragment E in rabbits. When admixed with fibrin degradation products the cell proliferative activity is abolished (17). However, most of our attempts to raise an antibody to fibrin fragment E have been hampered by the similarity of the fragment between species, particularly the human, mouse and rat. Accordingly, our antibodies have been made using alternative approaches. 1) Using synthetic peptides to the regions after the enzyme cleavage points to immunise mice and rats. Several polyclonal and monoclonal antibodies have been made using this method. 2) Screening of a synthetic antibody library made in Fd phage. It is likely that the structure is so well conserved because of the important role the fragments play in

stimulating biological activities. Using a human synthetic antibody library it is possible to screen for antibodies to the conserved regions which may be of importance in the biological activity.

Accordingly there are provided antibodies and antibody-like reagents which are able to block the binding of fibrin fragment E to its receptor. The antibodies could be monoclonal antibodies and the antibody-like reagents could be obtained by selection and screening of libraries of single chain antibody fragments in vitro. Other agents capable of binding to fibrin fragment E or the receptor could also be used.

Fibrin fragment E active site:

The techniques described below, using a phage epitope display library, can be used to give information on fibrin fragment E component peptide sequences within the active site that bind to the receptor on the cell surface. In other words, these sequences mirror the receptor structures to which they bind. These sequences are the basis for drug analogues, such as synthetic peptides and their mimics, that can activate or block the receptor.
The increase in cell proliferation seen in pathological lesions, such as the atherosclerotic plaque, may be initiated by the binding of fibrin fragment E to its cell surface receptor. Knowledge of the cell receptor could lead to the production of a competitive analogue and thus a potential new pharmaceutical approach to controlling cell proliferation responses. There are only a few changes to amino acid sequence between the human, rat and mouse and thus it may be that one of, or a combination of, these changes may be relevant to the blocking activity of the antibodies directed against the whole fragment E.

The initial approaches to stimulating or blocking the effect of the active site on human fibrin E using polyclonal antibodies have been both aided and limited by species differences. These differences have given rise to the immune reactions that have formed the antibodies of interest. There are known to be only a few differences to amino acid sequence between the human, rat and mouse molecules.

Antibodies also show the presence of a distinctive degradation pathway in macrophages.

METHODS

Receptor studies

Digoxygenin Labelling of Fibrin Fragment E

327mg of digoxygenin ester, dissolved in 8.18µl DMSO was added to 1mg of fibrin fragment E in 1 ml of phosphate buffered saline (PBS) and incubated at room temperature for 2hours. This was then dialysed against PBS (3 changes of 1l) to remove the unreacted ester and DMSO. Detection of the labelled protein was then carried out using anti-digoxygenin alkaline phosphatase raised in sheep (Boehringer).

Ligand blot

Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung cells (HEL) were cultured in a 25cm^2 flask (Nunc) until confluent (approximately 3x10^6 cells). The cells were then floated off the flask using PBS with 2% EDTA and slight scrapping with a rubber policeman. The cells were centrifuged for 5min at 3,000rpm and resuspended in hypotonic shock solution, sonicated for 30min and then centrifuged and resuspended in 1ml of PBS. 100µl of the cells was then added to 35µl of SDS containing gel loading dye (8%SDS, 40%glycerol, 12.5% 0.5M Tris-glycine buffer pH 6.8 and 10µg of bromophenol blue), and heated to 95°C for 5min.

50µl of the cells in loading dye were applied to gradient polyacrylamide gels (3-20%) and electrophoresed for 5 hours until the dye reached the base of the gel. The gel was then washed several times in Tris buffered saline containing 0.5% Triton X-100 to remove the SDS. The gel was then blotted on to PVDF membrane (Millipore) using a Bio-Rad blotting system in Tris glycine buffer (25mM Tris and 192mM glycine).

The blotting membrane was blocked using 5% bovine serum albumin in Tris buffered saline (TBS). The blot was then incubated overnight at 37°C with digoxygenin labelled fibrin fragment E (100µg in 100ml of TBS). The membranes were washed three times in TBS 0.5% Tween 20 for 10 minutes and incubated for 1 hour with sheep anti-digoxygenin antibody conjugated to alkaline phosphatase. The membrane was washed, 3 times, again with TBS Tween followed by development with nitro blue tetrazolium, bromo-chloro-indolyl-phosphate in sodium bicarbonate buffer ($NaHCO_3$ 100mM, $MgCl_2$ 10mM) to visualise the position of the receptor containing band.

Membrane Immunoblotting

Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung cells (HEL) were cultured in a $25cm^2$ flask (Nunc) until confluent (approximately $3x10^6$ cells). 100mg of digoxygenin labelled fibrin fragment E was added to the cells and incubated at 37°C. Cells were harvested by rinsing with PBS and then floated off the flask using PBS with 2% EDTA and slight scraping with a rubber policeman. The cells were centrifuged for 5min at 3,000rpm and resuspended in hypotonic shock solution for 15 min, centrifuged and resuspended in 1ml of PBS. 100µl of the cells was then added to 35µl of SDS containing gel loading dye (8%SDS, 40%glycerol, 12.5% 0.5M Tris glycine buffer, pH 6.8 and 10µg of bromophenol blue), and heated to 95°C for 5min.

50µl of the cells in loading dye were applied to gradient polyacrylamide gels (3-20%) and electrophoresed for 5 hours until the dye reached the base of the gel. The gel was then washed several times in Tris buffered saline containing 0.5% Triton X to remove the SDS. The gel was then blotted on to PVDF membrane (Millipore) using a Bio-Rad blotting system in Tris glycine buffer (25mM Tris and 192mM glycine).

The blotting membrane was blocked using 5% bovine serum albumin in Tris buffered saline (TBS). The blot was then incubated overnight at 37°C for 1 hour with rabbit anti fibrinogen antibody (Dako). The membranes were washed three times in TBS 0.5% Tween 20 for 10 minutes and incubated for 1 hour with goat anti rabbit antibody conjugated to alkaline phosphatase. The membrane was washed, 3 times, again with TBS Tween followed by development with nitro blue tetrazolium (NBT), bromo-chloro-indolyl-phosphate (BCIP) in sodium bicarbonate buffer ($NaHCO_3$ 100mM, $MgCl_2$ 10mM pH 9.8) to visualise the position of the receptor containing band.

Cell Immunohistochemistry

Chick fibroblasts were cultured in a $25cm^2$ flask (Nunc) until confluent (approximately $3x10^6$ cells). The cells were washed in PBS and then trypsinised and diluted to $3x10^5$ cells per ml in Dulbecco modified medium with 10% foetal calf serum. 50µl of the cells suspension was plated into each well of a Nunc culture well slide. The cells were incubated overnight to allow adhesion and recovery from the passaging. Digoxygenin labelled fibrin fragment E was added to a final concentration of 8µg per ml. Control wells contained unconjugated digoxygenin and PBS.

The cells were rinsed after 2 hours with PBS then washed 3 times in TBS followed by one wash in distilled water. The cells were then incubated for 2 hours with sheep anti-digoxygenin alkaline phosphatase. The slides were then washed three times in TBS and developed with

NBT and BCIP in bicarbonate buffer pH 9.8. The slides were air dried and then mounted for observation of membrane staining under the microscope.

Cell Binding Assays

Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung cells (HEL) were cultured in a $25cm^2$ flask (Nunc) until confluent (approximately $3x10^6$ cells). The cells were trypsinised and resuspended at concentration of $1.3x10^5$ cells/ml. 200µl of this cell suspension was added to each well of a 96 well culture plate (Nunc). The cells were incubated overnight at 37°C to allow adherence and recovery. The cells were then divided into three sections, digoxygenin controls, PBS controls and digoxygenin labelled fragment E tests. Increasing concentrations of digoxygenin and digoxygenin labelled fibrin fragment E were applied from 0-30µg per well. The plates were incubated for 6 hours at 37°C under culture conditions.

The plates were emptied of media and washed three times with PBS. The cells were then incubated for 2 hours with sheep antidigoxygenin alkaline phosphatase and developed with p-nitrophenyl phosphate in bicarbonate buffer pH 9.8. The plates were read on a Titertek Multiscan plate reader at 405nm. High absorbance indicating binding of the digoxygenin labelled fibrin fragment E to the cells (fig 4)

Antibody production

Polyclonal antibodies were raised to fibrin fragment E using the following immunisation protocol.

Rabbits and rats were immunised with fibrin fragment E, made by thrombin treatment of fibrinogen fragment E (Diagnostica Stago). 50µg of fibrin fragment E in 0.5ml of PBS was mixed with 0.5ml of Friends complete adjuvant. The rabbits were immunised intramuscular and the rats intraperitoneal. The animals were then boosted at 4 and 8 weeks later with 50µg of a long fibrin digest, which was shown to contain only fibrin fragment E, in incomplete Freunds adjuvant. The animals were then bled and the antisera tested for reactivity on blots of fibrin degradation products.

Blocking assays

The rabbit and rat antisera were mixed with active fibrin degradation products. These were applied to the CAM with a positive control (fibrin degradation products) and a negative control (the antibody alone). The CAM was processed for incorporation of tritiated thymidine. Results showed that the fibrin degradation products were active and that the rat and rabbit antisera to fibrin fragment E removed the activity (4)

The Phage Library

Rat and rabbit polyclonal antibodies were raised against purified human fibrin fragment E as described previously (4). All secondary antibodies were purchased from commercial sources (Sigma Chemicals). A sample of the 2 x 108 clone 15 amino acid peptide gene VIII library as described by Scott & Smith (1990) (27), was obtained as a phage suspension from George Smith (University of Missouri Columbia, USA). 20 ml of this phage suspension was infected into mid-log K91 cells, which were IPTG induced when the OD reached approximately 0.9 resulting in the formation of 6.6 x 109 tetracycline-resistant transductants. These were amplified by growth in one litre of 2 x TY medium containing 20 mg tetracycline/ ml for 24 hours at 37°C with shaking.

Before precipitation of the phage, the cultures were first spun for 10 minutes at 8,000 RPM at 4°C to remove bacteria. The supernatant was kept, and PEG 6000 20% (w/v), 0.5 molar NaCl, added resulting in a final PEG concentration of ~4% (w/v). The cultures were then

cooled on ice for 1 hour and centrifuged for 20 minutes at 10,000 RPM at 4°C. The phage were resuspended in 10ml of Tris buffered saline.

Selection of Phage

Selection of phage from the library was carried out using a procedure based on the biopanning method of Parmley & Smith (1988) (9). 1 ml of each polyclonal antibody in PBS at dilutions from 1: 200 to 1: 1,000 was added to a 60 mm petri dish (Nunc) and incubated in an orbital incubator at room temperature overnight to allow antibody adhesion. The next day 5mls of blocking solution (PBS, 0.5% Tween, 5 % skimmed milk), was added to each dish, in order to block any sites not adhered to by the polyclonals. The dishes were then left to incubate in an orbital incubator for 1 hour at room temperature, and then washed 5 times in PBST (PBS, 0.5% Tween).

100µl of the gene VIII library was added to each dish, and the dishes incubated at room temperature for 1 hour, in an orbital incubator. The unattached phage were then poured away and the dishes washed 5 times in PBS-Tween. Phage which remained bound to the immobilised polyclonals were recovered from the antibodies by the addition of 800µl 0.1 M HCl (pH. 2.2 with glycine), and allowed to incubate at room temperature for 15 minutes. 48µl of unbuffered 2 M Tris base was then added to each dish to neutralise the acid.

For round 1 biopanning, the contents of each dish were added to 3ml cultures of mid-log E. coli K91, and allowed to incubate at room temperature for 10 minutes. 12 ml of LB medium (10g Tryptone, 5g yeast extract, 10g NaCl in 1 litre H_2O, pH 7.5) was then added to each culture. Tetracycline was also added to a concentration of 0.2 mg/ml, and the cultures incubated at 37oC (225 RPM) for 40 minutes. Additional tetracycline was then added, to a final concentration of 20 mg/ml and the cultures allowed to grow for 16-20 hours at 37°C (225 RPM). Phage input and output titres were determined by plating and incubation on LB agar dishes.

A second round of amplification was carried out as for the first, except that 100µl of phage recovered from the first rounds of biopanning was used against the blocked polyclonals. A third round of biopanning was also carried out except that 100µl of phage recovered from the second round of biopanning was used against the blocked polyclonals.

During rounds 2 & 3 negative controls, consisting of K91 cells, with no added phage, were used to demonstrate that the K91 stocks had not acquired tetracycline resistance by another means i.e. plasmid transfer. A fourth round of amplification was carried out as for the third except that 100µl of phage recovered from the second round of biopanning was used against polyclonals from a different species, i.e. phage recovered after three rounds of biopanning against the rabbit polyclonals, was used against the rat polyclonals, and phage recovered after three rounds of biopanning against the rat polyclonals was used against the rabbit polyclonals. This was to demonstrate the homogeneity of different polyclonals raised in two different species against the same antigen and to reject non shared target epitopes

Testing of Reactive Clones

Reactive clones were identified from the output of round 4 with an ELISA using a 1: 200 dilution of rat anti fibrin E polyclonal for coating one set of wells, and the second coated with a 1: 200 dilution of rabbit anti fibrin E polyclonal. Samples of the selected phage clones were each run on PAGE and immunoblotted with the existing anti E antisera that were the basis of the selection process. The 60 selected reactive clones were combined into 3 groups, 1-20, 21-40, and 41-60, and samples mixed with Freund's complete adjuvant and used to immunise rats and rabbits. The resultant new antisera were tested initially by immunoblotting of PAGE of selected phage clone proteins and also human FDP. Then the rabbit antisera were tested for ability to block the stimulatory activity of human FDP on the chick CAM model. The assay

used was based on quantitative measurement of DNA synthesis in the CAM after exposure to control and test substances 18 h after application in liquid form to the whole "dropped" area of each CAM (4,29). This assay is a measure of changes in CAM vascularity (30). Anti phage antisera, raised by relatively short term immunisation of rabbits, were used at a 1/2 dilution and admixed with FDP used at a concentration of approximately 1.95 mg/ml diluted before use to 1/10. Controls included buffer only and antiserum only groups of CAMs. After filter sterilisation, 0.3 ml was added onto each CAM dropped surface.

RESULTS

Use of the Phage Library

The gene VIII phage display peptide library was relatively simple to use. Extra care needs to be taken at the stage of biopanning, when the phage are being removed from the plate, to infect the log K91 cells. The original protocol allowed five minutes for this infection procedure, but ten should be allowed to ensure successful transformation of the bacteria. If this stage is unsuccessful then the phage will not infect the bacteria, and the bacteria will not have acquired resistance to the antibiotic, and there will be no phage output. It is vitally important to ensure that the stock of bacteria have not acquired resistance to the antibiotic from a source other than the phage, especially when in an environment where other workers are using antibiotics. A simple control experiment at each stage (i.e. K91s with no phage in media with tetracycline), will determine if the stock of bacteria is still suitable to use. Another stage which requires care is the phage precipitation step. At this stage it is important to cool the supernatant before centrifugation. This simply ensures a higher yield of phage as increasing numbers are precipitated at lower temperatures.

Testing of Reactive Clones

The sequence of checks to ensure that the selected clones are genuinely reactive with the 2 antisera that formed the basis of that selection is summarised in Figure 5. Two examples of these stages are shown. First, the demonstration of binding of rat anti phage 1-10 combined to individual phage clone proteins is shown in Figure 2. Second, the binding to human fragment E sized bands by several of the new antisera raised is seen in another immunoblot (Fig 6).

Demonstration of Blocking of FDP Angiogenic Activity

Figure 7 illustrates one experiment where rabbit anti phage 1-20 does not appear to block the stimulatory effect of FDP after admixture, but rabbit anti phage 21-40 and 41-60 do inhibit the stimulation. Repeat experiments have now been completed a further 2 times for the latter two antibodies with similar inhibition. A further experiment with anti phage 1-20 has shown some inhibition and this antibody is still under investigation. Antibody alone and buffer alone controls have no effect. Rat antibodies have not yet been tested. The first 3 antibodies raised against individual clones, clones 42, 43, and 44, have been found to be inhibitory (Fig 8), but the phage inserts have not yet been sequenced. Many earlier experiments performed in the same way with such polyclonal rabbit antibodies against synthetic fibrin E epitopes have not shown such inhibition.

Mapping the site for blocking:

To locate the active site on fibrin fragment E, we have used existing polyclonal anti E antibodies in combination with a Fd phage random epitope display combinatorial library. This is a genetically engineered culture of phage that randomly displays on its cell surface short sequences of fifteen amino acids. This property can be used as a selective binding tool, and phage which bind to the selection agent, often a chosen antibody, can be grown on as pure clones and eventually be analysed for the sequence information.

The epitopes on the human fragment E molecule are detectable via two existing rat and rabbit polyclonal blocking antibodies which are used to select phage clones that display epitopes common to the site on the molecule. Sequential selection and amplification has yielded 60 clones of phage. Samples of these clones were combined in three groups for immunisation. Rabbit antiserum against clones 1 to 20 shows a little blocking activity against the cell stimulatory activity of fragment E. Polyclonal antisera raised against 21 to 40, and 41 to 60 detect fragment E bands on immunoblots, and more crucially, consistently block all the angiogenic activity of fragment E. Further antisera against individual clones from most of group 41-60 have now been made. Blocking activity has been reproduced with anti clones 41, 42, 43, 44, 45 but not clone 49, 53. Sequence information has been obtained from clones 45, 49 sequencing of the other clones is ongoing.

Sequences obtained for clone 45
CRAHSFGSPRPLPVV
SRAHSFGSPRPLPVV
CRAHSFVSPRPLPVV
QPDPHLMMWKLPGFP

Sequence obtained for clone 49
ALSKRPVGRPRVCTG

The diversity of possible sequences which are present in a random peptide library, represent a potentially wide range of affinities. Selection procedures isolate a number of clones from the many millions in the library and some of these isolated sequences should share sequence elements.

The epitopes on the human fragment E molecule that are detectable via the two existing rat and rabbit polyclonal blocking antibodies must include epitopes within or adjacent to the active site for stimulating angiogenesis. These antibodies have been used to select phage clones that display epitopes common to this site on the molecule. Sequential selection and amplification has yielded 60 clones of phage. Polyclonal antisera raised against 21 to 40, and 41 to 60 detect fragment E bands on immunoblots, and more crucially, substantially block the angiogenic activity of fragment E (Fig 7). These antisera have been made as further selection tool. Furthermore, antisera against individual phage have blocking activity (Fig 8).

The sequence information for the finally selected epitopes, derived from analysis of each phage clone DNA insert, can be used not only to locate the active site on the molecule, and to perpetuate blocking antibodies, but also to synthesise large quantities of short peptides and short peptide analogues. These can be tested for competitive blocking activity for human fragment E. Such peptides and analogues are potential therapeutic agents in the longer term for blocking the cell stimulatory effects of fibrin fragment E in vivo in a potentially wide variety of pathologies (30) without the attendant risks of interfering in clotting or fibrinolysis. Our previous work has shown that admixture of blocking antisera to fibrin E will inhibit the angiogenic effect of experimental mouse wound extracts (31) and extracts of proliferative types of human atherosclerotic plaques (21). We have not yet attempted inhibition in vivo. Sustained delivery in vivo to inhibit, for example, wound healing should be more readily achieved by administration of small peptides than polyclonal antisera from another species.

The sequence information for the finally selected epitopes, derived from analysis of each phage clone DNA insert, has been used to locate parts of the active site on the molecule. The sequence information can also be used to synthesise large quantities of short peptides. These can be tested for competitive blocking activity for human fragment E. Such peptides and analogues are potential therapeutic agents.

Knowledge of the location of the active site, even though limited to a few short segments of the protein molecule, allows further exploration of non-immunogenic adjacent areas, within the

known molecular structure of fibrin E, which may influence biological activity. Synthetic peptides and analogues of such regions are likely to provide further experimental agents that are potential drugs.

Therefore the present invention should be considered to encompass any type of molecule with the property of interaction with fibrin fragment E or its cell surface receptor to modulate cell proliferation at pathological sites, including angiogenesis, and any method for generating such molecules. Further, although the above description has specifically instanced applications to human disease, the invention is equally applicable to other animal species.

Fibrin fragment E plays an important role in atherosclerosis. We have identified the major component of plaque extracts that stimulate cell proliferation as fibrin fragment E. We have now demonstrated using ligand blots of chick fibroblast membrane preparations that fibrin fragment E mediates cell proliferation by binding to the cell surface. The receptor is of particular interest in smooth muscle cells, endothelium and macrophages, which play an important role in atherosclerotic lesions. Alternative therapeutic approaches might include the development of drugs which regulate the expression of the fibrin fragment E receptor in target cells and the use of antisense RNA. Fibrin fragment E analogues or other agents which interact with the fibrin fragment E receptor, or regulate its expression, could also be used in stimulating or inhibiting wound healing or in treating cancer. For example, in invasive tumours the coagulation system is active at the tumour edge and the importance of angiogenesis in tumour development is well known.

These applications of the present invention should not be considered as defining limits of the present invention as it will be obvious to those skilled in the art that numerous other applications of the present invention are possible. In addition, the methods for generating antibodies or antibody-like reagents or other binding molecules of the present invention should not be considered as defining limits of the methods of this invention; indeed, the present invention should be considered to encompass any type of molecule with the property of recognising the fibrin fragment E or its receptor and any method for generating such molecules. Furthermore, although the above description has specifically instanced applications to human disease, the invention could equally be applied to other animals.

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FIGURE LEGENDS

Fig.1: Ligand blot of chick fibroblast SDS-treated membrane preparation
An SDS-PAGE mini-gel (10% acrylamide) was subjected to electroblotting and the blot was then challenged with digoxigenin-labelled fibrin fragment E which in turn was detected with anti-digoxygenin antibody/ alkaline phosphatase conjugate. Alkaline phosphatase activity was detected by standard mehods and showed the presence of a band corresponding to a molecular weight of 66kDa. Lanes on the left panel - whole FDP conjugate;
lanes on the right panel - fibrin fragment E conjugate.

Fig. 2: Immunoblot of cell membrane previously challenged with fragment E

Fig 3A and 3B: Staining of cells using digoxygenin-labelled fibrin fragment E

Fig. 4: Graphs of binding assays for Chick fibroblast, 3T3 cells and Cos7 challenged with digoxygenin labelled fragment E.

Fig. 5: An immunoblot of SDS PAGE of phage numbers 1 to 10 detected with rat anti phage 1-10 combined. At least 8 out of the 10 phage clones contain coat proteins reactive with the rat anti 1-10.

Fig. 6: An immunoblot of SDS PAGE of human fibrin degradation products. Each strip has been exposed to a different antibody.

1 rat anti E
2 rat anti 1-20
3 rat anti 21-40
4 rat anti 41-60
5 rabbit anti E
6 rabbit anti 1-20
7 rabbit anti 21-40

There is a difference in pattern of human FDP band staining between the two species, reflected by both anti fragment E antibodies and the antibodies against phage clones selected by both these initial antibodies. The rabbit antisera immunostain more strongly bands between 45-50 kD which represent plasmin derived fragments E1, E2 and E3. Rat antibodies immunolabel more strongly higher molecular weight combinations of DDE, DED, and DE. (both E, and D and E combinations are angiogenic).

Fig. 7: The effect of admixture of 3 different rabbit anti phage antibodies with stimulatory FDP. Anti phage 1-20 does not have an effect in this experiment, whilst anti phage 21-40 and 41-60 have abolished the stimulatory effect of FDP.

Fig. 8: Admixture of anti phage antibodies against selected phage clones 42, 43, and 44 abolishes the stimulatory effect of FDP. Student's t test on log transformed data shows a significant ($P<0.05$) increase in DNA synthesis compared with the buffer only control group by FDP. The 3 anti phage groups are significantly different from the FDP group, but not from the control group.

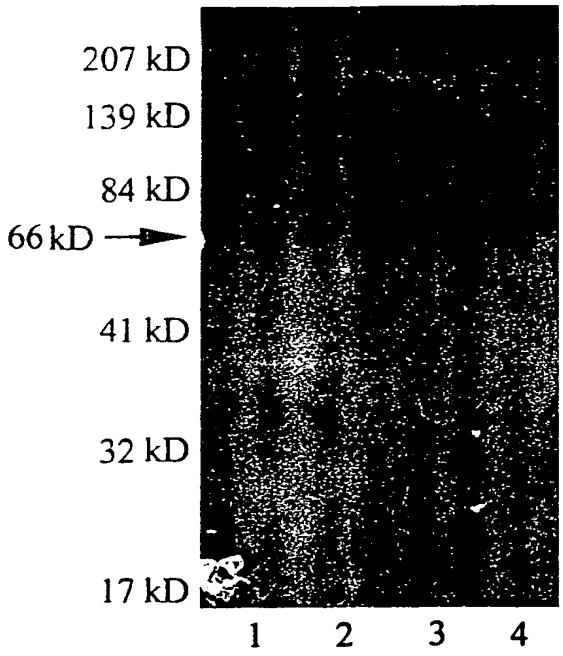


Figure 1 showing ligand blot of several cell types with digoxygenin labelled fibrin fragment E.
1. Chick fibroblasts.
2. Mouse 3T3 fibroblasts.
3. Cos 7 monkey kidney cells.
4. Human embryonic lung cells.

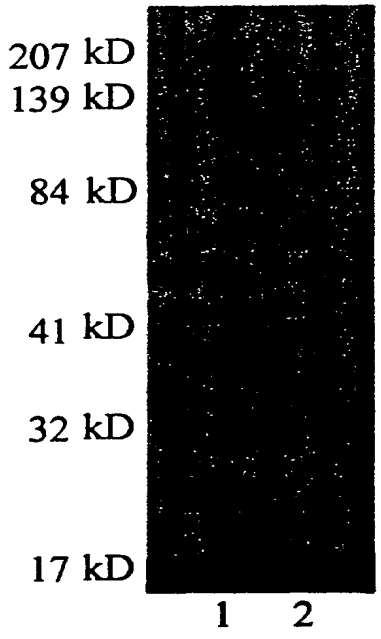


Figure 2 showing immunoblot of fragment E bound to chick fibroblast cell membrane, giving a band of molecular weight approximately 122Kd, consistent with 55Kd for fragment E and 66Kd for the cell receptor calculated from figure 1.

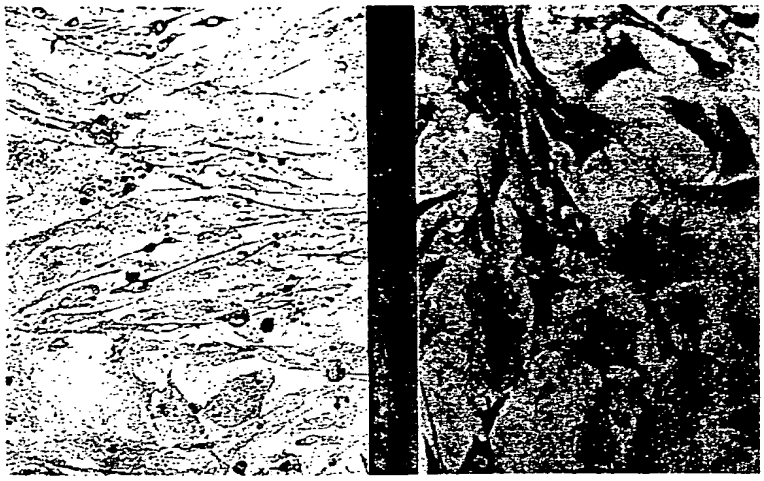


Figure 3A: Chick fibroblasts in culture challenged with digoxigenin labelled fibrin fragment E.
Left side: control Right side: test

Figure 3B: Higher magnification of 3A test to show enhanced surface membrane staining of cells ⟶

Fig4A

Binding of Digoxygenin Labelled Fibrin Fragment E to Chick Fibroblast Cells

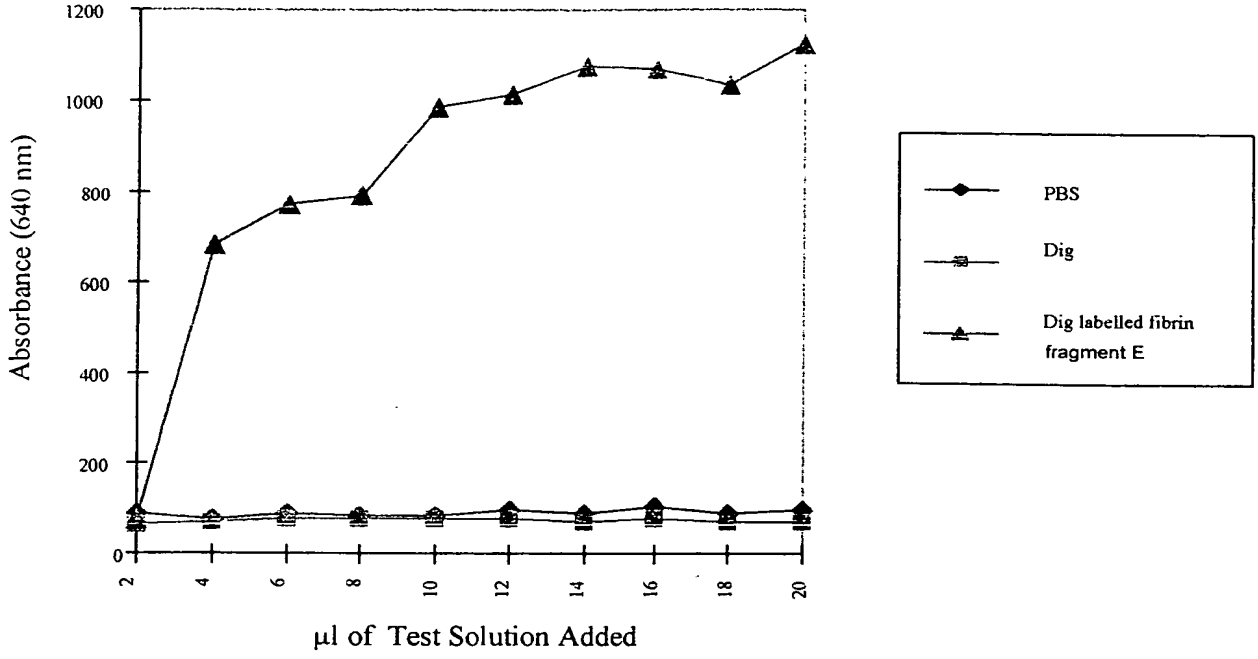


Fig4B

Binding of Digoxygenin Labelled Fibrin Fragment E to Mouse 3T3 cells

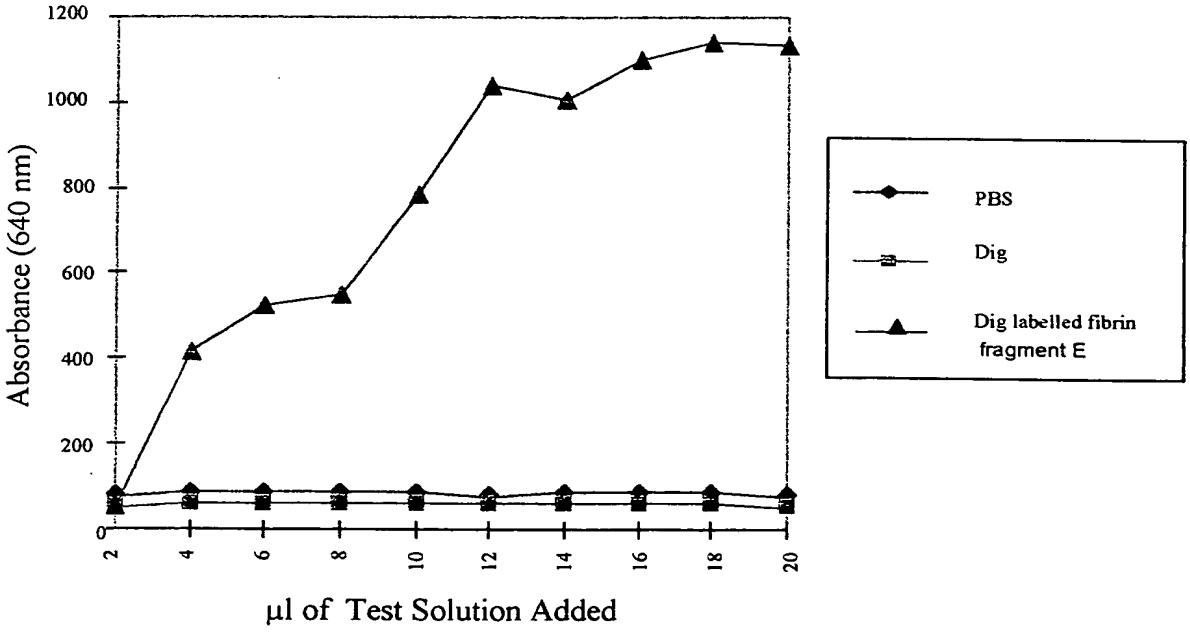
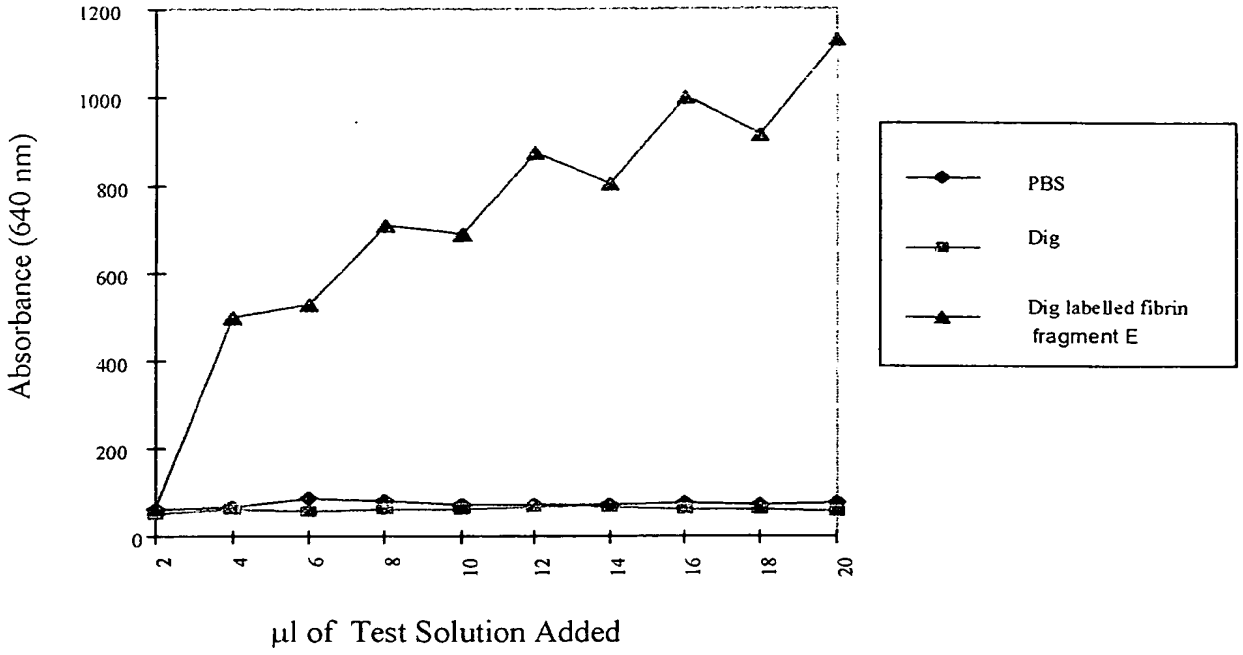
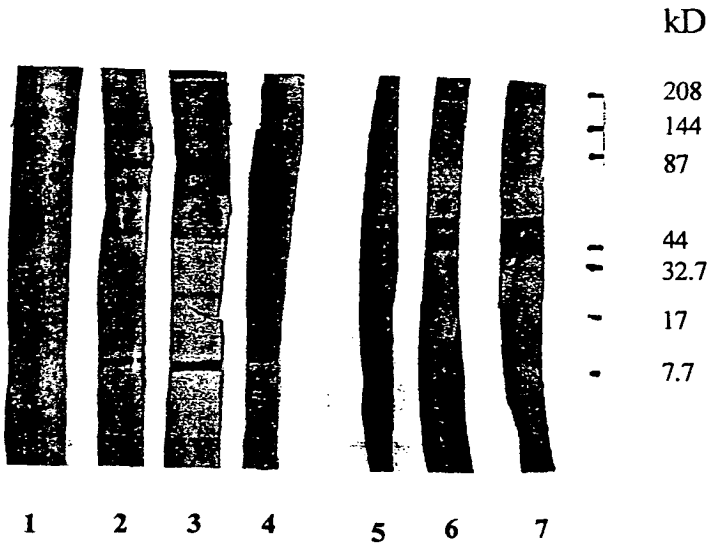


Fig 4C

Binding of Digoxygenin Labelled Fibrin Fragment E toCos7 cells



[Fig 6]


kD
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44
32.7
17
7.7
1
2
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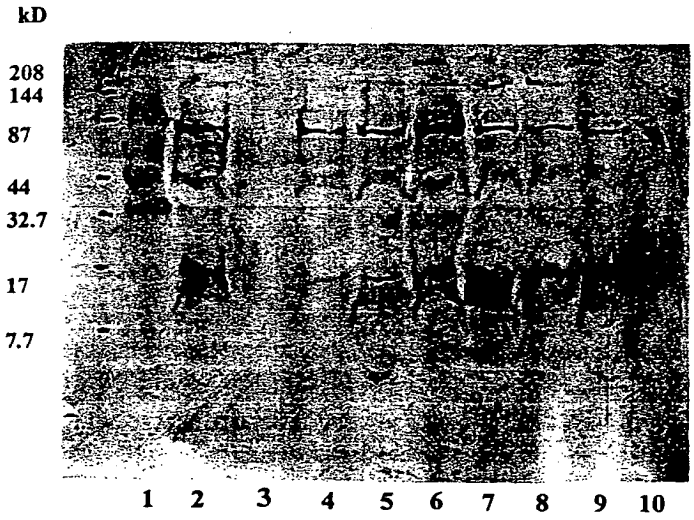
6
7

[Fig 5]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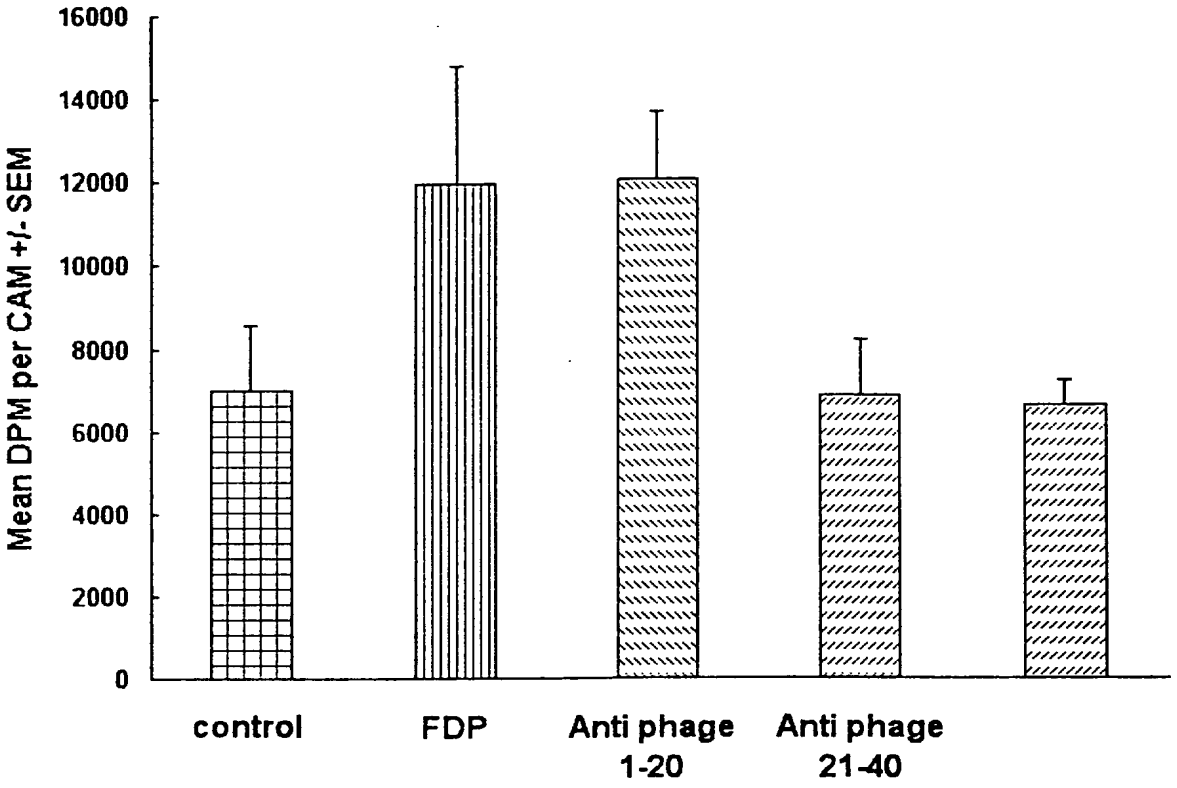
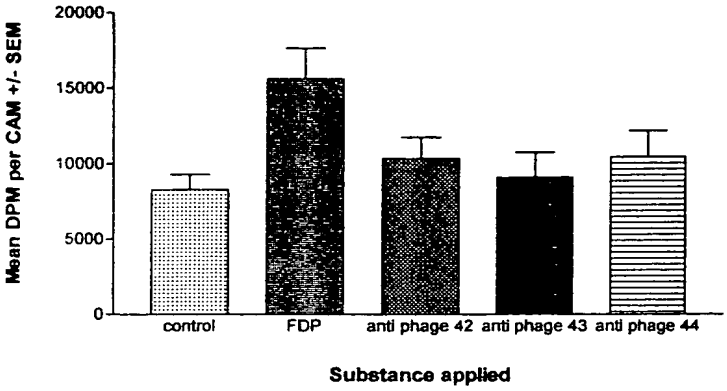

16000
14000
12000
10000
8000
6000
4000
2000
0
Mean DPM per CAM +/- SEM
control
FDP
Anti phage 1-20
Anti phage 21-40

Fig.7

[Fig 8]


Mean DPM per CAM +/- SEM
20000
15000
10000
5000
0
control
FDP
anti phage 42
anti phage 43
anti phage 44
Substance applied

PCT/GB00/02197

4.6.00

Newburn Ellis